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Role of Myeloid-Derived Suppressor Cells in tumor-associated pregnancy

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ABSTRACT

Cancer progression is dependent, in part, on interactions between tumor cells and the host microenvironment. During pregnancy, physiological changes occur that include inflammation and reduced immunity, both of which can promote tumor growth. Accordingly, tumors are observed to be more aggressive and to have greater proclivity toward metastasis during pregnancy. In this work, myeloid-derived suppressor cells (MDSC), a population of heterogeneous and pluripotent cells that can down-regulate immune responses during pathological conditions, were studied in the context of mouse and human gestation. The gene expression profile of mouse MDSC has been shown to differ in pregnant and virgin mice, and the profile in pregnant animals bears similarity to that of MDSC associated with the tumor microenvironment. Common induced genes include Fibronectin1 and Olfactomedin4, which are known to be involved in extracellular matrix remodeling and tissue permissiveness to tumor cells implantation. Our observations suggest that mouse MDSC may represent a shared regulatory mechanism of tissue permissiveness that occurs during the physiological state of gestation and tumor growth.

Pregnancy-associated changes in immunosuppressive myeloid cell activity have also been studied in humans. We show that CD33⁺ myeloid cells isolated from PBMC (peripheral blood mononuclear cells) of pregnant women are more strongly immunosuppressive on T cells than CD33⁺ cells removed from non-pregnant subjects. During murine gestation, decreased natural killer (NK) cell activity is responsible, at least in part, for the increase in experimental metastasis. However, although peripheral blood NK cell numbers and cytotoxicity were slightly reduced in pregnant women, neither appeared to be regulated by CD33⁺ cells. Nevertheless, based on its observed suppression of T cell responses, the CD33⁺ PBMC subset appears to be an appropriate myeloid cell population to study in order to elucidate mechanisms of immune regulation that occur during human pregnancy. Our findings regarding the immunosuppressive function of CD33⁺ cells and the role of NK cells during human pregnancy are consistent with the notion that changes in the function of the immune system participate in the constitution of a permissive soil for tumour progression.

Keywords: cancer, metastasis, immune system, Myeloid-Derived Suppressor Cells, pregnancy

INTRODUCTION

1. Tumor-host interactions

Metastasis is a multistage process that constitutes the primary cause of cancer related death. It requires tumor cells to detach from the primary tumor mass, migrate toward and eventually invade lymphatic or blood vessels, survive within the circulation, attach to the endothelium of distant organs, penetrate the endothelial barrier and establish new tumor colonies. The process is highly inefficient since less than 0.1% of tumor cells that penetrate the circulation will eventually generate metastatic colonies (1)(2).

Throughout this process, interactions occur between cancer cells and the host microenvironment, including the immune system. It is well established that interactions between tumor cells and the host tissue stroma play a key role in determining whether and how any given solid malignancy will develop. In most cases, tumor cells take control of stromal cell functions for their own benefit and dictate the rules of engagement to the host tissue microenvironment. The host immune system also participates in tumor growth regulation through the recognition and destruction of cancer cells. However, cancer cells display properties that help them avoid immunosurveillance, including selection of tumor-cell variants and subversion of the immune system (3).

Observations of this cross-talk between tumor cells and the host microenvironment that may seem to “prepare” a tissue for subsequent invasion and secondary growth, have led to the concept of “pre-metastatic niches”. Distal host tissues thus appear to adopt functional features that facilitate tumor cell implantation, survival and division. An important question to address is whether these niches are exclusively tumor-associated or whether they reflect a more general response of the organism to a variety of potentially pathological or physiological stimuli, as may occur during pregnancy.

2. Metastasis during pregnancy

Cancers that develop during pregnancy usually have a worse prognosis than those that arise in non-pregnant women of a comparable age. One of the best documented example is breast cancer. Women who develop breast cancer during pregnancy (or within one year of giving birth) have tumors that are often very aggressive with high metastatic proclivity (4)(5). Although increased hormone production may contribute to tumor progression, 80% of breast cancers in this age group are hormone receptor negative, suggesting that mechanisms other than hormone exposure are required to promote cancer aggressiveness. These include potential changes in the immune system that occur during gestation or the peripartum.

Two of the most important changes during pregnancy are reduced immunity, to allow proper fetal implantation, and induction of inflammation. These physiological changes may help install permissive niches for tumor growth. However, little is known about the host factors during pregnancy that determine whether or not a disseminating tumor cell will be able to spawn a new colony once it reaches its metastatic site.

3. Immunity & pregnancy

During normal pregnancy, various immunological changes are observed. Humoral (antibody-mediated) immunity of the mother is required to protect her and her fetus from the environment while the fetus develops its own immune system, imposing adaptation on the mother to accommodate its presence (6). The human placenta contains a high number of immune cells, such as macrophages, regulatory T-cells and dendritic cells. NK cells constitute a markedly predominant population and T lymphocytes are particularly abundant. Many diverse modulators such as sex-hormones, maternal antibodies, the complement system and cytokines are also recruited to the placental interface (6).

Modifications of the immune system that are involved in preventing rejection of the fetus include absence of classical class I MHC molecules on the surface of fetal cells, hyporesponsiveness of immune cells as a result of Indoleamine 2,3-dioxygenase (IDO) activity, silencing of alloreactive T-cells by regulatory T-cells and expression of NK and T-cell modulating HLA-G molecules (1).

In addition to immunological changes, pregnancy is associated with an inflammatory response that varies according to the trimester. Inflammation is required for fetal implantation during the first trimester, becomes slightly reduced during the second trimester which is a quiescent fetal growth period and increases during the final trimester to prepare delivery (7)(6).

Local changes are supported by systemic changes during pregnancy. Thus, reduction in T-cell and B-cell numbers and activity are detectable in maternal blood. In addition, NK cells appear to be particularly important in early pregnancy, but after 20 weeks of gestation, their numbers decrease in maternal blood and display reduced activity. The decrease in T-cell, B-cell and NK cell functions and numbers is accompanied by an increase in innate immunity, which may offset their downregulation. An increase in the number of phagocytic immune cells, including monocytes, dendritic cells and neutrophils, is observed in maternal blood from as early as gestational week 20 all the way to week 36 (8).

A population of myeloid cells called myeloid derived suppressor cells (MDSC) is suspected to play a critical role in modulating the immune system in situations as distinct as tumor growth and pregnancy.

4. MDSC and their potential role in pregnancy

MDSC are a population of heterogeneous, immature and pluripotent myeloid cells that are precursors of granulocytes, macrophages and dendritic cells (9). They were recently shown to accumulate in mice in various pathological conditions, including cancer, infection and autoimmune diseases, where differentiation of immature myeloid progenitor cells is partially blocked. These immature myeloid cells are able to down-regulate immune responses and thereby facilitate tumor progression (10)(11).

MDSC are induced and/or activated by multiple proinflammatory mediators produced by tumor or host cells in the tumor microenvironment (Figure 1) (10)(11). Once activated, their production of immune suppressive factors, such as Arginase 1, inducible Nitric Oxide Synthase (iNOS), reactive oxygen species (ROS) and nitric oxide (NO) is increased, thereby inhibiting T cell and NK cell functions (10)(11).

These immature progenitor cells express receptors that allow their identification in various tissues. In mice, MDSC are characterized as CD11b⁺Gr-1⁺ cells (12). Under pathological conditions, MDSC found in blood and tissues outside the bone marrow display a granulocytic (80-90%) or monocytic phenotype (10-20%) with different surface markers. The Gr-1 marker is common to the two subsets and is also known as the Ly-6G/Ly-6C epitope. The equivalent population of cells is much more complex to define in humans, where their features appear to vary according to the tissue microenvironment or their differentiation state. Human peripheral blood MDSC have been characterized by expression of CD11b, CD15, CD33, CD66b, CD124 and low expression levels of HLA-DR (11)(13).

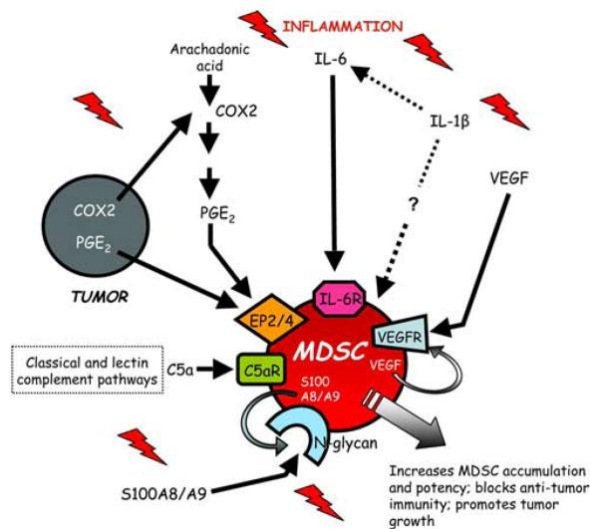


Figure 1: MDSC are induced and/or activated by multiple proinflammatory mediators. MDSC accumulate in the blood, bone marrow, lymph nodes, and at tumor sites in response to pro inflammatory molecules produced by tumor cells or by host cells in the tumor microenvironment. These factors include PGE₂, IL-1β, IL-6, VEGF, S100A8/A9 proteins, and the complement component C5a.

MDSC accumulate in the context of tumor growth, inflammation and the physiological condition of pregnancy. Observations made in the laboratory have shown that tumor-bearing pregnant mice develop metastasis more aggressively than virgin counterparts (14)(1)(4). Remarkably, this was shown to be the case irrespective of tumor type or model. In-depth investigation of the putative mechanisms involved revealed that pregnancy induces accumulation of MDSC that infiltrate a broad range of tissues, to help generate a permissive soil for tumor cell engraftment. These MDSC effectively block T and NK cell activity and were shown to be responsible for accelerated and diffuse metastatic tumor growth in pregnant mice (15)(1)(4).

SPECIFIC AIMS OF THE STUDY

Since we know that pregnancy-associated changes promote tumor progression in mice, at least in part due to the contribution of MDSC, our specific aims are the following:

1. To evaluate whether the gene expression profile of MDSCs during pregnancy differs from that in non-pregnant (or virgin) animals, with the goal of elucidating how this MDSC population augments its immunosuppressive properties and why it accumulates during the physiological state of gestation. This may allow development of tools to neutralize their function and to reverse their phenotype in order to restore a competent immune response.
2. To determine whether the functions of MDSC are different in human blood cell isolates obtained from pregnant and non-pregnant women. We will try to transpose the knowledge obtained from mouse models to human pregnancy and assess whether MDSC accumulate more and are functionally more efficient than in non-pregnant women.

RESULTS & DISCUSSION

1. Gene expression profiles of MDSC extracted from pregnant mice display features that may augment permissiveness for tumor progression

1.1. Results

1.1.1. Enrichment of MDSCs

To assess the expression profile of MDSC during pregnancy, a protocol to isolate and enrich this population had to be optimized. Eight 16-day-pregnant C57BL/6 mice (equivalent to 30th weeks of human pregnancy) and six non-pregnant C57BL/6 mice were euthanized and splenocytes isolated. After MACS-sorting of the Gr1^{High}-Ly-6G positive cells on two columns, the enrichment of the cell suspension was assessed by flow cytometry using anti-Gr1, anti-CD11b and anti-CD45 antibodies. The percentages of CD45⁺ CD11b⁺ and Gr1^{High} populations were doubled from the initial splenocyte populations (Figure 2: 28% and 37% MDSC before sorting, to 75% and 79% after the enrichment for virgin and pregnant mice, respectively).

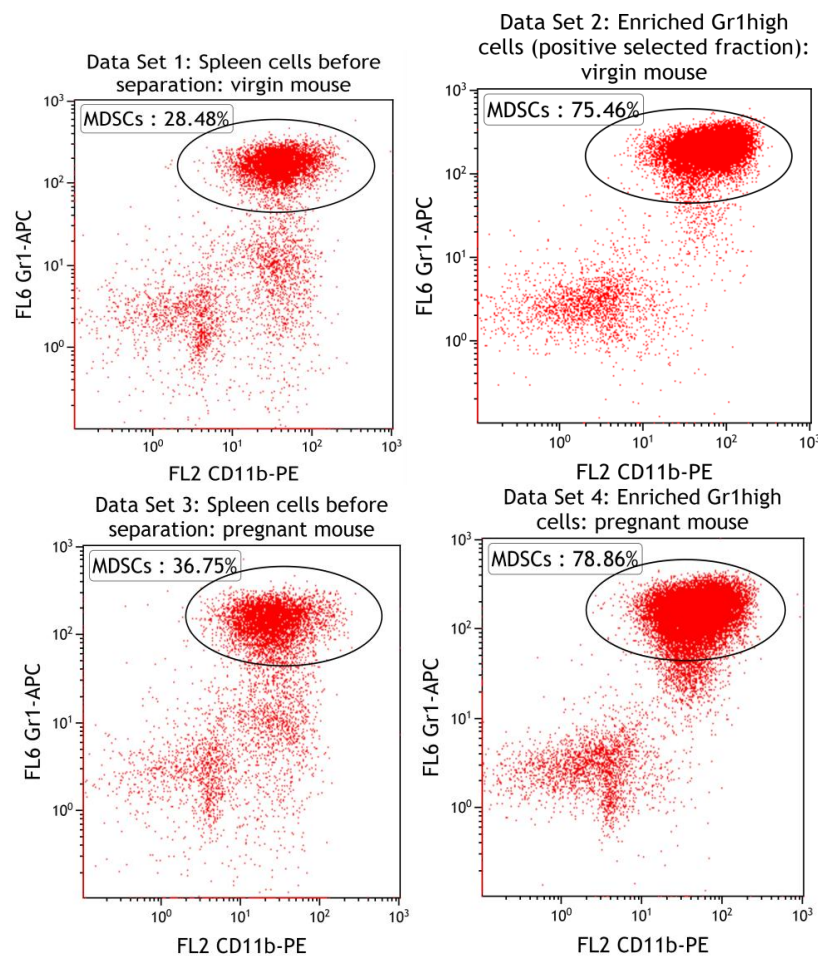


Figure 2: Representative data of flow cytometry analysis of splenocyte fractions isolated from pregnant and virgin mice, before and after Gr1^{High}-Ly-6G positive cell sorting. These results were gated on the leukocyte CD45⁺ cell population. Cells were fluorescently stained with anti-CD45-FITC, anti-CD11b-PE and, anti-Gr1-APC.

1.1.2. Hybridization on Affymetrix microarrays

After MDSC isolation and extraction of their total RNA (Qiagen RNeasy® Mini Kit), microarrays were performed at the Genomics Technology Facility of Lausanne (GTF). Four RNA samples extracted from MDSC of pregnant (2) and virgin mice (2) were initially prepared and transferred to the GTF. Because RNA quality had to be optimized, only one of each RNA extractions (1P and 1V) was used in the initial experiment for hybridization to an Affymetrix Mouse Gene 1.0ST array.

A second experiment was done with 2 batches of MDSC extracted from pregnant (2) and virgin (2) mice. After analysis of the whole dataset (6 samples), a list of 81 relevant probes appeared to be differentially expressed (69 up- and 12 down-regulated – Table 1) by cells extracted from pregnant mice compared to those from virgin mice. The probes were sorted according to log2FC. Among these genes, several are known to be involved in facilitating tumor progression or linked to poor prognosis in cancer, including Fbn1 (16) (17), Prg4 (18), Abca13 (19), Itgb2l (20), Cldn1 (21), Fcnb (22), Olfm4 (23), or Saa3 (24). Some of these genes were also linked to inflammation, including chitinases, Lbp (25), or Rgs1 (26).

Gene symbol	Gene description	log2FC
<i>Up-regulated genes</i>		
Fbn1	fibronectin 1	2.058
Prg4	proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)	1.90441
Lipg	lipase, endothelial	1.87258
Inhba	inhibin beta-A	1.72573
Olfml2b	olfactomedin-like 2B	1.68228
Abca13	ATP-binding cassette, sub-family A (ABC1), member 13	1.64987
Itgb2l	integrin beta 2-like	1.60919
4930438A08Rik	RIKEN cDNA 4930438A08 gene	1.59185
Cldn1	claudin 1	1.55661
Chi3l7	chitinase 3-like 7	1.55356
Lbp	lipopolysaccharide binding protein	1.52177
Fcnb	ficolin B	1.43574
Olfm4	olfactomedin 4	1.43459
Saa3	serum amyloid A 3	1.42808
Ica1	islet cell autoantigen 1	1.42732
Chi3l3	chitinase 3-like 3	1.41066
Syne1	synaptic nuclear envelope 1	1.32292
Serpinb10-ps	serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 10	1.31097
Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	1.29145
Thbs1	thrombospondin 1	1.28941
Cd177	CD177 antigen	1.2728

Crispld2	cysteine-rich secretory protein LCCL domain containing 2	1.25273
Olr1	oxidized low density lipoprotein (lectin-like) receptor 1	1.25165
Gypa	glycophorin A	1.21583
Ankrd22	ankyrin repeat domain 22	1.2136
Orm1	orosomucoid 1	1.20731
Mki67	antigen identified by monoclonal antibody Ki 67	1.20162
Kif11	kinesin family member 11	1.1933
Slc4a1	solute carrier family 4 (anion exchanger), member 1	1.19264
Syne1	synaptic nuclear envelope 1	1.17749
Pde7b	phosphodiesterase 7B	1.17694
Arg1	arginase, liver	1.16789
Gca	Grancalcin	1.15974
Alox15	arachidonate 15-lipoxygenase	1.15291
Ncam1	neural cell adhesion molecule 1	1.14901
Bub1	budding uninhibited by benzimidazoles 1 homolog (<i>S. cerevisiae</i>)	1.14137
Lta4h	leukotriene A4 hydrolase	1.13636
Ccna2	cyclin A2	1.11758
Prc1	protein regulator of cytokinesis 1	1.09922
1100001G20Rik	RIKEN cDNA 1100001G20 gene	1.09714
Aspm	asp (abnormal spindle)-like, microcephaly associated (<i>Drosophila</i>)	1.0954
Acpl2	acid phosphatase-like 2	1.0902
Camp	cathelicidin antimicrobial peptide	1.08793
Adpgk	ADP-dependent glucokinase	1.08539
Slco4c1	solute carrier organic anion transporter family, member 4C1	1.07311
Rhou	ras homolog gene family, member U	1.0719
Prr11	proline rich 11	1.0688
Depdc1a	DEP domain containing 1a	1.06875
Ly6g5b	lymphocyte antigen 6 complex, locus G5B	1.06418
Cpne2	copine II	1.06135
Cebpe	CCAAT/enhancer binding protein (C/EBP), epsilon	1.06022
Cxcr1	chemokine (C-X-C motif) receptor 1	1.05865
Kif18b	kinesin family member 18B	1.05534
Plk1	polo-like kinase 1 (<i>Drosophila</i>)	1.04735
Gpr97	G protein-coupled receptor 97	1.04475
Dach1	dachshund 1 (<i>Drosophila</i>)	1.04457
Plscr1	phospholipid scramblase 1	1.04081
Pf4	platelet factor 4	1.03449
Aff2	AF4/FMR2 family, member 2	1.03064

Nt5e	5' nucleotidase, ecto	1.03045
Rnf125	ring finger protein 125	1.02841
Serpinb2	serine (or cysteine) peptidase inhibitor, clade B, member 2	1.02762
Aldh3b2	aldehyde dehydrogenase 3 family, member B2	1.02673
1700055N04Rik	RIKEN cDNA 1700055N04 gene	1.02239
Trp53inp2	transformation related protein 53 inducible nuclear protein 2	1.0212
Depdc1a	DEP domain containing 1a	1.0118
Anln	anillin, actin binding protein	1.01177
Tinagl1	tubulointerstitial nephritis antigen-like 1	1.01132
2310005G13Rik	RIKEN cDNA 2310005G13 gene	1.00302
<i>Down-regulated genes</i>		
Rgs1	regulator of G-protein signaling 1	-2.02762
Apol7c	apolipoprotein L 7c	-1.57539
Ccl24	chemokine (C-C motif) ligand 24	-1.19042
Slamf8	SLAM family member 8	-1.17702
Tsen15	tRNA splicing endonuclease 15 homolog (S. cerevisiae)	-1.10621
Rgs13	regulator of G-protein signaling 13	-1.08822
Zdhhc2	zinc finger, DHHC domain containing 2	-1.08753
Dip2c	DIP2 disco-interacting protein 2 homolog C (Drosophila)	-1.08659
Il1a	interleukin 1 alpha	-1.08264
Bcl2a1c	B-cell leukemia/lymphoma 2 related protein A1c	-1.05915
Ilgp1	interferon inducible GTPase 1	-1.02036
Tnfsf18	tumor necrosis factor (ligand) superfamily, member 18	-1.00411

Table 1: Differentially expressed genes between MDSC extracted from pregnant and virgin mice. In red, are 5 genes selected for qRT-PCR validation of the microarray data.

1.1.3. Validation of microarray results by qReal-Time RT-PCR

Among genes up-regulated in MDSC extracted from pregnant mice, five of them (in red in the table 1) were chosen for qReal-Time RT-PCR validation, with a $\log_2FC \geq 1.41$ (= at least 2 fold more expressed). MDSC were extracted from 3 new virgin and 4 16-day pregnant C57BL/6 mice, and mRNA was prepared for analysis by qRT-PCR (Figure 3). The 5 selected genes are involved in matrix remodeling, tumor progression and regulation of innate immunity. Four of them appeared to be significantly overexpressed ($p < 0.05$ or $p < 0.01$), confirming the initial hybridization data.

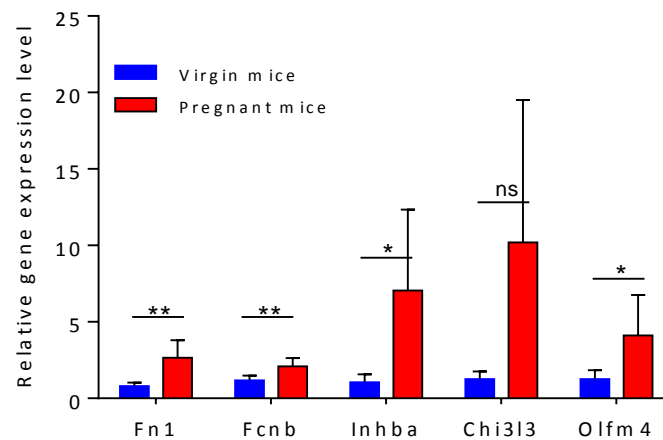


Figure 3: qReal-Time RT-PCR validation of selected up-regulated genes identified by microarray analysis.

1.1.4. Comparison of MDSC genes expressed in pregnant and tumor-bearing mice

To validate the involvement of these genes in tumor development, four healthy and four tumor-bearing mice (TBM) were used for splenocyte extraction, MDSC sorting and qRT-PCR assessment of the expression of the five selected genes. The same expression profile observed in the comparison between pregnant and virgin mice was observed when MDSC derived from TBM were compared to their healthy animal counterparts (Figure 4), consistent with the functional implication of these genes in tumor growth.

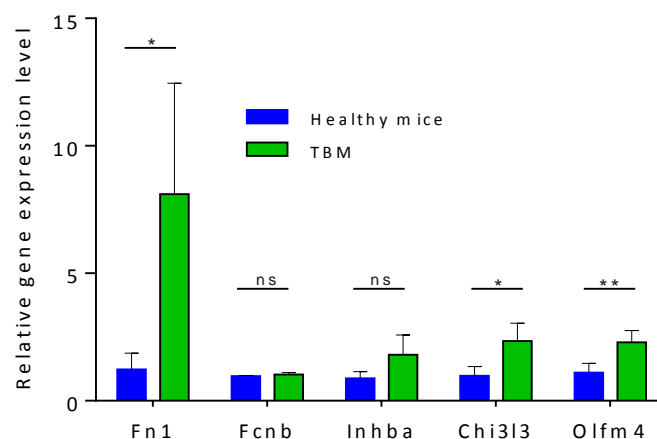


Figure 4: qReal-Time RT-PCR of genes expressed by MDSC extracted from TBM compared to healthy mice.

The mannose receptor, metalloprotease 9 (MMP9), interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF α) mRNA were also shown to be increased in MDSC extracted from pregnant mice compared to virgin ones, as well as from TBM as expected (Figure 5). Indeed, these four

genes that encode a receptor, a protease and two cytokines, are known to facilitate tumor progression through their overexpression in tumor-associated macrophages (TAM).

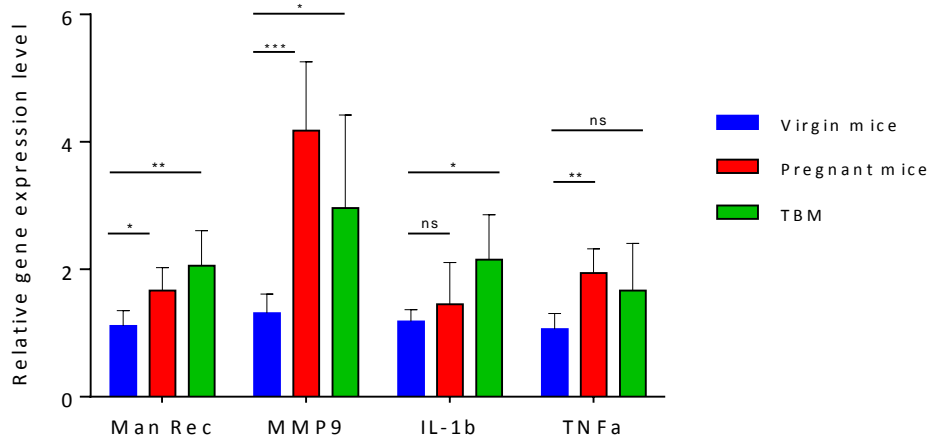


Figure 5: qReal-Time RT-PCR of genes expressed by MDSC extracted from pregnant and TBM compared to virgin-healthy mice.

1.2. Discussion

As described, gene expression profiling was assessed in two separate experiments using 2 different RNA extractions. In these gene expression profile studies aimed at further characterizing pregnancy-associated MDSC, the arrays showed different patterns of expression: no change, slight or important increase or decrease in gene expression. First, we observed that the transcriptome of pregnancy-associated MDSC was significantly different from that of MDSC in virgin mouse tissues. The second observation was the similarity between the gene expression profiles of MDSC from pregnant and tumor-bearing mice.

Herein, we focused our study on MDSC genes that were overexpressed in both pregnant compared to virgin and tumor burdened compared to tumor-free mice. However, we cannot rule out that many genes that are marginally upregulated in pregnant mouse MDSC are also functionally important in the tumor microenvironment. Silencing of several genes expressed in MDSC may also be important during pregnancy but these genes were not assessed at this stage in this work.

The selected differentially expressed genes in MDSC isolated from virgin versus pregnant mice have various functions. A summary of some of their functions is provided below.

Olfactomedin-4 (OLFM4) gene encodes the antiapoptotic protein GW112 or the G-CSF-stimulated clone 1 protein. The OLFM4 protein is expressed during normal myeloid-specific lineage differentiation (27). The suspected role of OLFM4 in tumorigenesis is mainly related to its ability to regulate apoptosis, cell cycle (cell proliferation by promoting the S-phase transition) and cell adhesion (through interactions with lectins and cadherin)(28). Perturbations in OLFM4

expression decrease cell adhesion and alter cell-to-extracellular matrix interactions, with the possible consequence of cellular transformation and metastasis (27). Furthermore, OLFM4 protein has been shown to be highly expressed in several types of tumors, especially in gastrointestinal cancers (25) and gastric *H. pylori* infection where it downregulates T cell immunity and leads to chronic inflammation. OLFM4 is therefore an excellent candidate molecule to provide an explanation for some of the functions observed in pregnancy-associated MDSC.

Ficolin B (FCN2) also known as Fcn2, ficolin-2, ficolin-beta, collagen/fibrinogen domain containing lectin. This gene product has carbohydrate binding and opsonic activity for apoptotic/necrotic cells. The mouse ficolin B corresponds to human M-ficolin, a leukocyte-associated form of ficolins (29). Mouse ficolin B is mainly expressed in the bone marrow and spleen, as well as by peritoneal macrophages, whereas M-ficolin has been localized to the surface of monocytes and identified in the secretory granules of neutrophils and monocytes (30). Human ficolin activates the lectin branch of the complement cascade when they bind to microbial targets. It also activates endothelial cells. In contrast, mouse ficolin B does not activate complement. Functions of this protein have still to be understood, in addition to its involvement in innate immunity.

Inhibin beta-A (INHBA) also known as activin beta-A chain, follicle-stimulating hormone-releasing protein. Activins and inhibins are cytokines involved in cell differentiation and proliferation. Activins, which belong to the TGF- β superfamily, are homo- and heterodimers composed of inhibin/activin betaA-subunit or betaB-subunit, whereas inhibins are heterodimers of an alpha- and one of the beta-subunits (31). In humans, the beta-A subunit gene expression has been shown to be higher in both local and metastatic breast carcinoma than in normal tissue. But the effects and mechanisms of action of inhibin on cancer cells are largely unknown (32). IL-1 β is known to stimulate activin A and INHBA mRNA expression and is also an activator of MDSC.

In addition to its growth/differentiation factor function, INHBA acts as a hormone that negatively regulates pituitary FSH secretion. Serum levels of inhibin have been shown to be associated to the size of granulosa-cell tumors and can therefore be used as a marker for primary as well as recurrent disease of the ovary (33).

Fibronectin 1 (FN1) also known as migration-stimulating factor (MSF), an oncofetal isoform of Fibronectin. It is a glycoprotein present in soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in the extracellular matrix (ECM). Fibronectin 1 is involved in cell adhesion and migration in processes that include embryogenesis, wound healing, blood coagulation, host defense, and metastasis.

The continuous and dysregulated remodeling of the ECM in tumors leads to the aberrant presence of numerous extracellular matrix components that are not usually found in normal tissues (34). In transformed cells and in tumors, the splicing pattern of Fibronectin pre-mRNA becomes altered, leading to an increased expression of oncofoetal isoforms, including MSF.

MSF is a truncated fibronectin with motogenic activity, relevant to cancer development. In addition to stimulating cell migration, FN1 enhances hyaluronan synthesis, proteolytic activity, angiogenesis and the preparation of premetastatic niches. Thus MSF contributes to the motile phenotype of neoplastic cells (34) and has a strong chemotactic activity for monocytes and tumor cells. MSF is produced by various cell types, including neoplastic cells, tumor-associated vascular endothelial cells and type M2-macrophages and TAM (34). In the present study, we have shown that MDSC from pregnant mice produce increased amounts of MSF.

Chitinase 3-like 3 (Chi3l3) is also known as secreted protein Ym1 and eosinophil chemotactic cytokine. In mammals, although endogenous chitin or chitin synthases do not exist, chitinase and chitinase-like proteins (CLP) are endogenously expressed and dysregulated in allergic disorders and a variety of diseases characterized by chronic inflammation and tissue remodeling. CLP lack chitinase activity and their roles have still to be investigated. Chi3l3 is rodent-specific CLP and is expressed in macrophages after stimulation with IL-4 or IL-13 in response to parasitic infection or allergic stimulation, and is induced by Th2 cytokines. Chi3l3 is also expressed in dendritic cells and mast cells. The protein is known to be chemotactic for eosinophils, T lymphocytes, and polymorphonuclear leukocytes (35). Even if Chi3l3 is only expressed in mice, the CLP family contains human members, which are known to be involved in a variety of inflammatory, remodeling, and neoplastic disorders (35).

The difference in Chitinase 3-like 3 gene expression between pregnant and virgin mice was not significant. One potential explanation is the large scattering of Chi3l3 expression values among pregnant mice MDSC and the small number of samples. The mean of the gene expression level is affected by these extreme values. Another limiting factor of these experiments lies in the step of MDSC purification and the risk that genes found to be overexpressed in pregnant mouse MDSC are not exclusively coming from this cell population. Although RNA was prepared following cell purification some residual cells unrelated to MDSC may still persist.

The functions in the tumor context of these selected overexpressed genes are largely unknown. However they appear to be promising candidates to help understand the role of MDSCs in pregnancy and tumorigenesis. OLFM4 and MSF seem to be particularly interesting in the context of tissue permissiveness, and further investigation that involve the use of knockout and other transgenic mouse models, should be performed in the future. Our experiments also suggest a role of the immune population of MDSC in ECM remodeling and cell adhesion, both of which play important roles in tumor progression.

A larger study comparing MDSC content from tumor-bearing and tumor-free animals was also performed as described above. The same upregulation of the selected genes was observed, confirming the involvement of MDSC in facilitating metastasis during pregnancy.

The final goal of this study is to provide clues as to how MDSC facilitate tumor growth and how pregnancy might provide a favorable immunological precursor state for tumor implantation.

Interestingly, additional data have revealed similarities between pregnant mouse MDSC gene expression and TAM. Macrophages undergo activation in response to environmental signals that lead to polarization into at least two subtypes: proinflammatory M1 macrophages and protumor M2 macrophages. Tumors have their own associated-macrophages, which undergo a wide spectrum of polarized activation states, and have the potential both to elicit tumor and tissue destructive reactions and to promote tumor progression (macrophage balance). In general, TAM from established tumors have properties of activated M2 cells, but not only so, and as such support cancer progression and metastasis. Therefore, TAMs are a key component of pathways connecting inflammation and cancer (36)(37)(38).

MDSC share several similarities with TAM, as both cell types secrete elevated levels of IL-10, are able to inhibit the T-cell response, and promote angiogenesis. Furthermore, and in distinction from TAM, a relevant immunosuppressive mechanism of MDSC is mediated by the concomitant expression of the inducible forms of both nitric oxide synthase and arginase, which are

considered to be hallmarks of M1 and M2 macrophages, respectively, highlighting the plasticity of these cells (36)(37)(38).

Two examples of common induced genes observed during this study, expressed by pregnant and tumor-bearing mouse MDSC, are the mannose receptor gene (or CD206) and MMP9, usually prominent markers of the M2-macrophage phenotype. Moreover, both types of MDSC overexpressed IL-1 β and TNF α , as do M1 proinflammatory macrophages, suggesting the plasticity described above: pregnancy may prepare MDSC to differentiate both into inflammatory macrophages and into TAM, even in the absence of tumor cells, consistent with the potential role of these cells to create a permissive microenvironment already under physiological conditions.

2. MDSC functions during human pregnancy

2.1. Results

2.1.1. Choice of the MDSC population

In mouse models, MDSC accumulate in organs and blood, and have more potent immunosuppressive activity in pregnant than in virgin mice. To address the possible variations of MDSC in human pregnancy and in tumor growth facilitation related to pregnancy, we applied all of the above techniques to isolating human MDSC. The first step was to identify a candidate myeloid cell subtype. Unlike the MDSC population in mice that is well characterized, the phenotype of human heterogeneous MDSC populations is still unclear. Human MDSC are often described as CD14⁺, CD33⁺, CD11b⁺, HLA-DR^{low} for the monocytic subset and CD15⁺, CD33⁺, CD11b⁺, HLA-DR⁻, CD66b⁺ for the granulocytic subset. Several pilot studies were required to optimize the identification of the MDSC in humans. We collected blood samples from final trimester pregnant women and isolated the peripheral blood mononuclear cell (PBMC) and the granulocyte layers by Lymphoprep (see materials and methods) in order to isolate the two subpopulations (monocytic and granulocytic) of MDSC.

We then extracted and sorted the CD11b⁺ and CD33⁺ cells in these two layers, as well as T and NK cells. We tested the immunosuppressive effects of CD11b⁺ and CD33⁺ cells on sorted T and NK cells (co-cultures followed by a T cell proliferation assay or by a NK cell cytotoxicity assay). Based upon the known literature (39) and on our preliminary results on different blood samples (Figure 6 and Figure 7), we identified the PBMC layer and selected CD33⁺ myeloid cells for pregnant versus non-pregnant comparison.

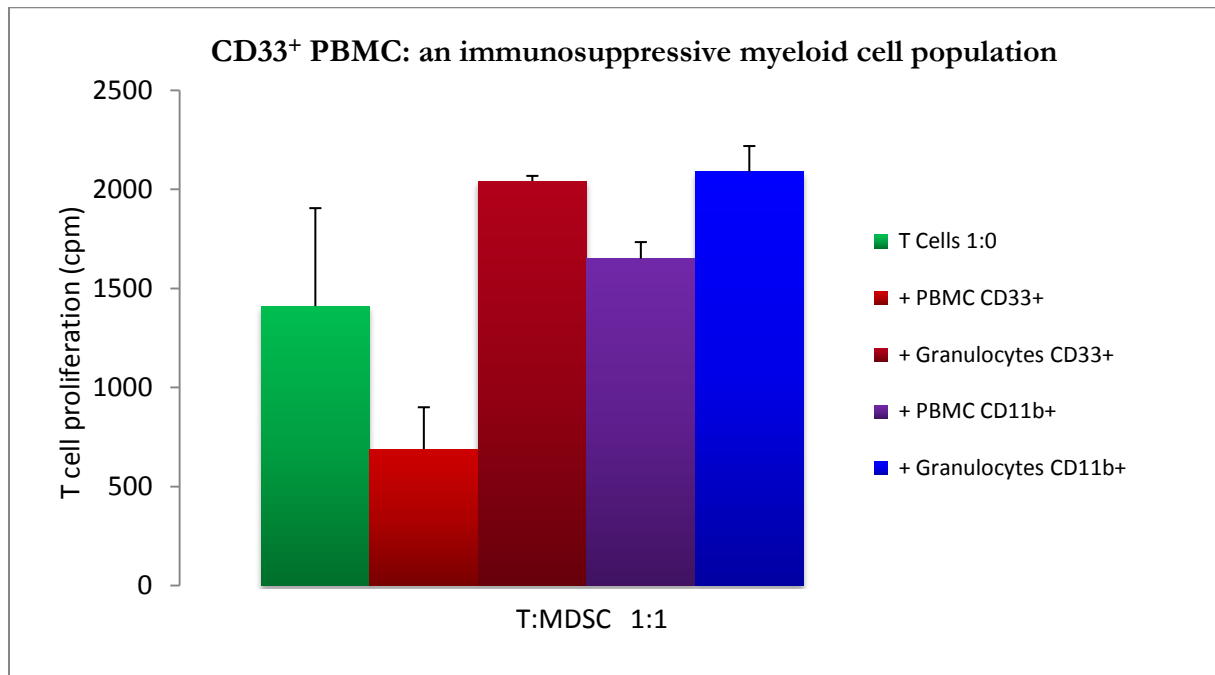


Figure 6: ³H-Thymidine T cell proliferation assay confirmed the immunosuppressive effect of the CD33⁺ PBMC co-cultured with T cells, whereas CD11b⁺ PBMC and CD33⁺/CD11b⁺ Granulocytes have no effect on T cell proliferation. Cells extracted from the blood of a non-pregnant woman. Proliferating T cells incorporate ³H-Thymidine.

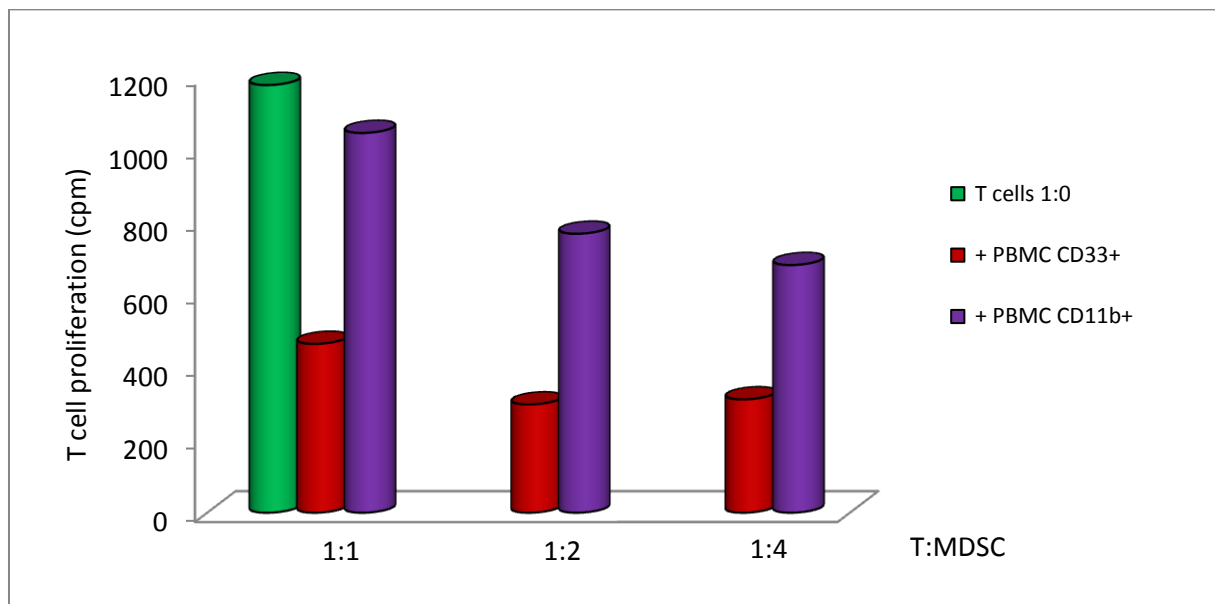


Figure 7: ³H-Thymidine T cell proliferation assay. Validation of the immunosuppressive effect of the CD33⁺ PBMC compared to the CD11b⁺ PBMC population. Cells extracted from the blood of a non-pregnant woman.

2.1.2. Immunosuppressive effect of CD33⁺ cells on T cells

After defining the cell population to study, we performed immunosuppressive function testing by co-culturing the selected CD33⁺ population and T cells (isolated from a single buffy coat). CD33⁺ PBMC isolated from the blood of eight 32-week pregnant women were compared to cells extracted from 8 non-pregnant women. The protocol for isolation and access to the donors were accepted by the ethical committee of our Institution.

A slight decrease in proliferation of T cells co-cultured with CD33⁺ cells from pregnant women versus non-pregnant women was observed (Figure 8). These first results were not significant possibly due to the large scattering of values, but they were encouraging enough to repeat the experiment with 8 new blood samples obtained from unrelated pregnant women. This second experiment (Figure 9) confirmed reduced T cell proliferation when co-cultured with CD33⁺ cell from pregnant women compared to non-pregnant women. Moreover, it was associated with a CD33⁺ dose-related decrease in T cell proliferation.

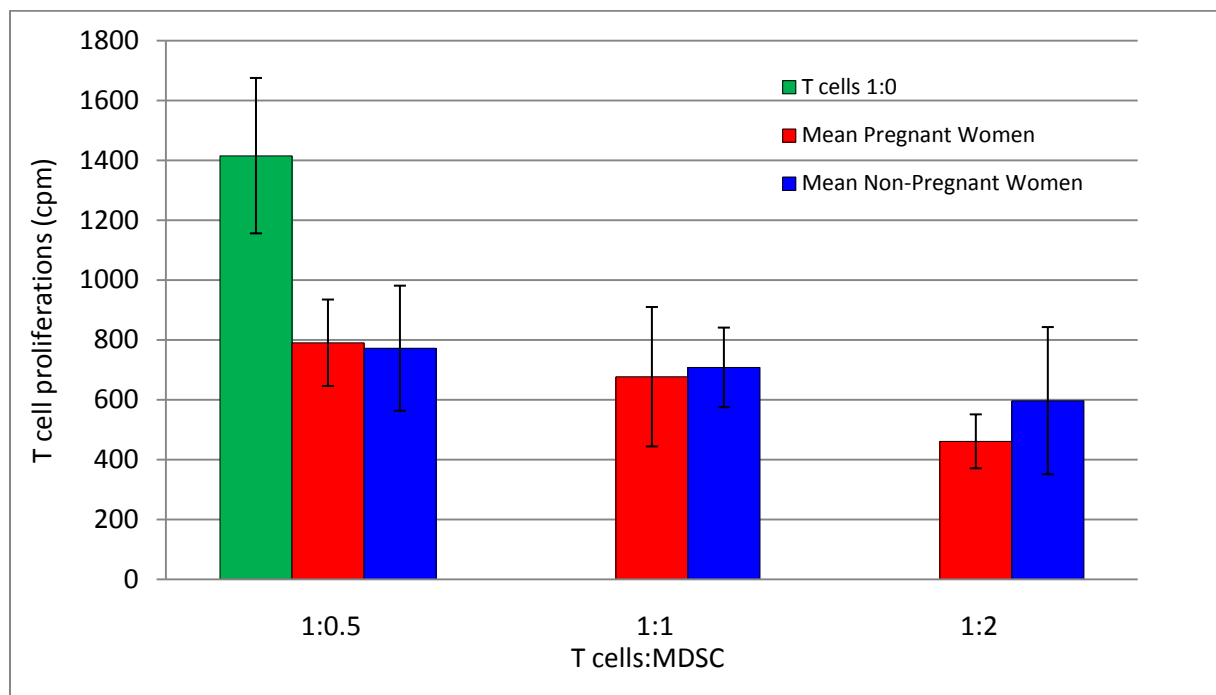


Figure 8: ³H-Thymidine T cell proliferation assay, first experiment.

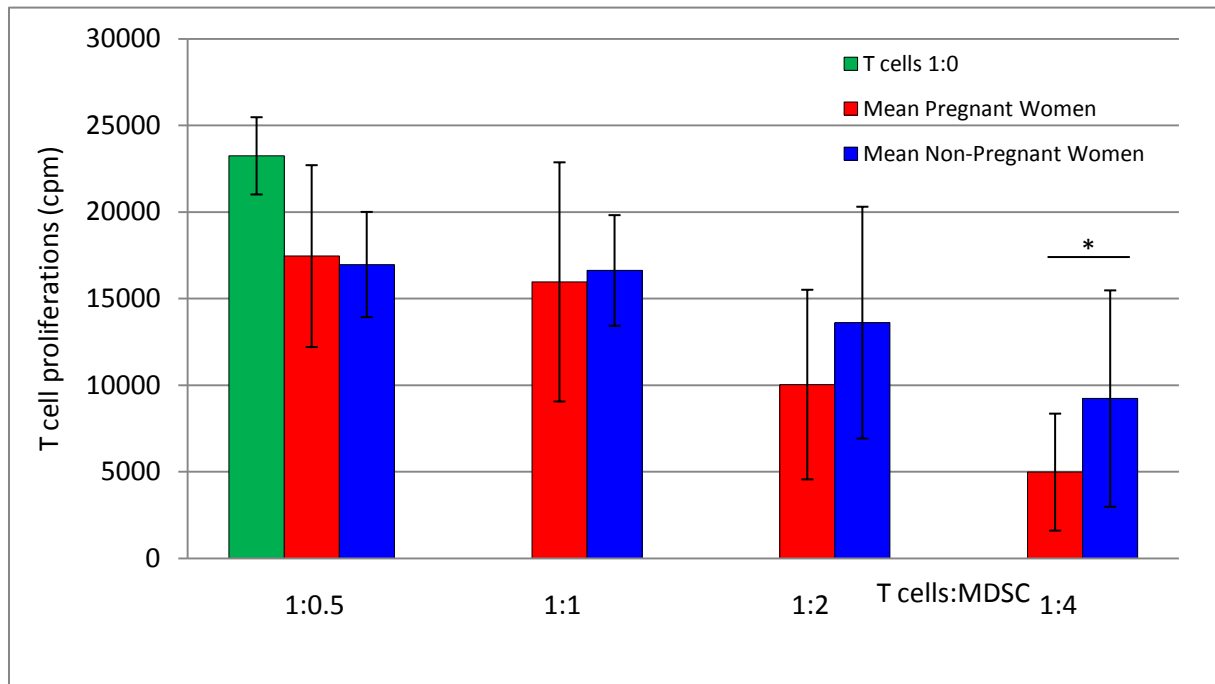


Figure 9: ^3H -Thymidine T cell proliferation assay, second experiment.

These experiments allowed us also to conclude that there was no significant change in the quantity of $\text{CD}33^+$ cells circulating in the blood of pregnant compared to non-pregnant women. We did not observe the same accumulation which was observed in mice, at least in circulating blood.

2.1.3. Immunosuppressive effect of $\text{CD}33^+$ cells on NK cells

The observations of Mauti and her colleagues (1) indicated that murine gestation increases the rate and the degree of tumor dissemination irrespective of the tumor cell origin. Moreover, they showed that decreased NK cell numbers and cytotoxicity lead to blunted local clearing of tumor cells and demonstrated that MDSC contribute to the reduced NK cell activity and corresponding enhancement of tumor dissemination. MDSC isolated from tumor-bearing animals have been shown to decrease NK cell activity in a cell contact dependent manner and to be responsible for increased tumor dissemination (1).

To better understand the role of MDSC in human pregnancy and how they function, we performed a NK-MDSC co-culture following by a cytotoxicity assay (see material and methods). NK cells were isolated from a single buffy coat. They were co-cultured for 18h with $\text{CD}33^+$ PBMC from blood samples of pregnant and non-pregnant women, activated with human IL-2 before being plated with target cells (K562 cell line) labeled with $^{51}\text{CrO}_4$. NK cells are known as one of the key effectors of tumor cells elimination. K562 tumor cell lysis induced by NK cells releases $^{51}\text{CrO}_4$ in the supernatant.

The result of these co-cultures did not show any direct effect of $\text{CD}33^+$ cells extracted from pregnant or non-pregnant women on NK cell cytotoxicity (Figure 10).

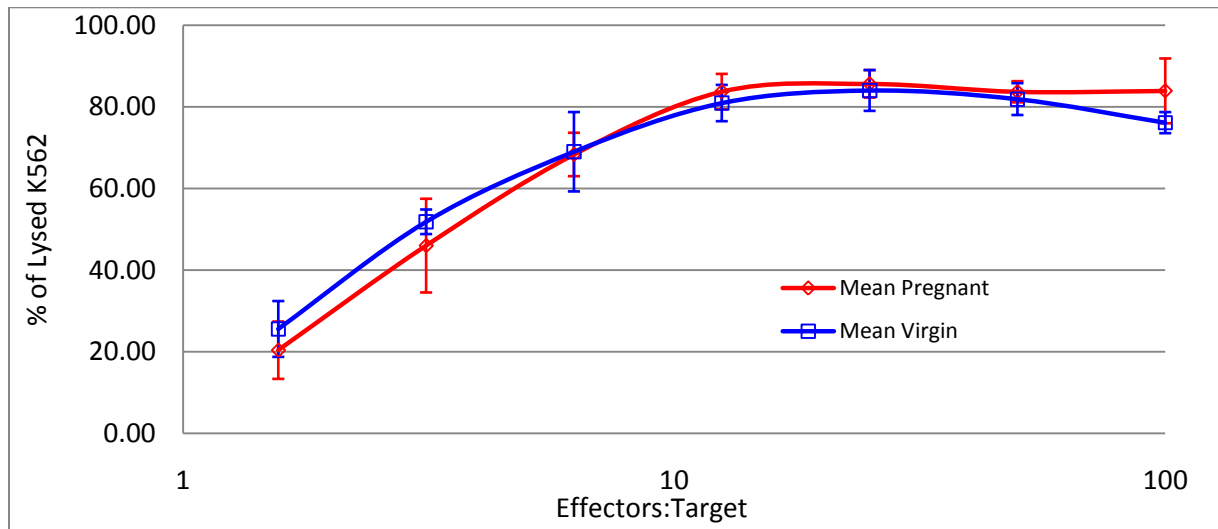


Figure 10: NK-CD33⁺ co-culture and cytotoxicity assay (mean of 4 32-week pregnant women blood samples compared to 4 non-pregnant blood samples).

2.1.4. NK cell ability to target tumor cells

The CD33 negative fractions of PBMC were used for the assessment of direct cytotoxicity of NK cells on K562 cells. The resulting graph was adjusted for NK cell proportions among CD33⁻ cells, as determined by flow cytometry. Interestingly, NK cells represented a lower percentage in the blood of pregnant than non pregnant women (Figure 11), consistent with the literature. However, NK cell cytotoxicity was not significantly different whether the NK cells were extracted from the blood of pregnant or non-pregnant women (Figure 12). These observations suggest that circulating NK cell activity is not differentially regulated during human pregnancy, but NK cells are present in lower quantities. The human CD33⁺ PBMC population, that bears similarity to the mouse MDSC population in terms of the down-regulation of the immune response, did not show a direct role in the regulation of NK cell function.

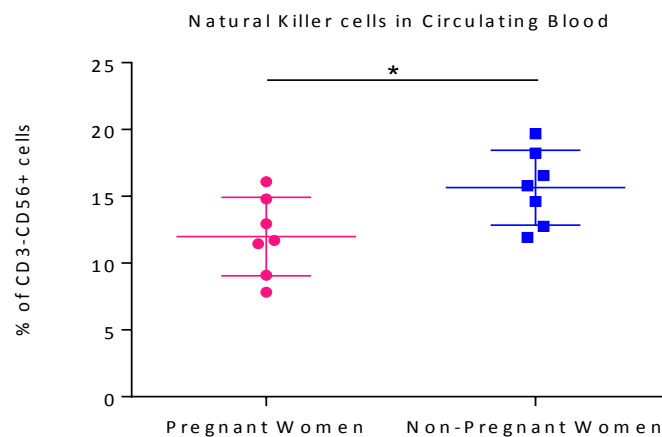


Figure 11: FACS quantification of NK cells in blood circulating.

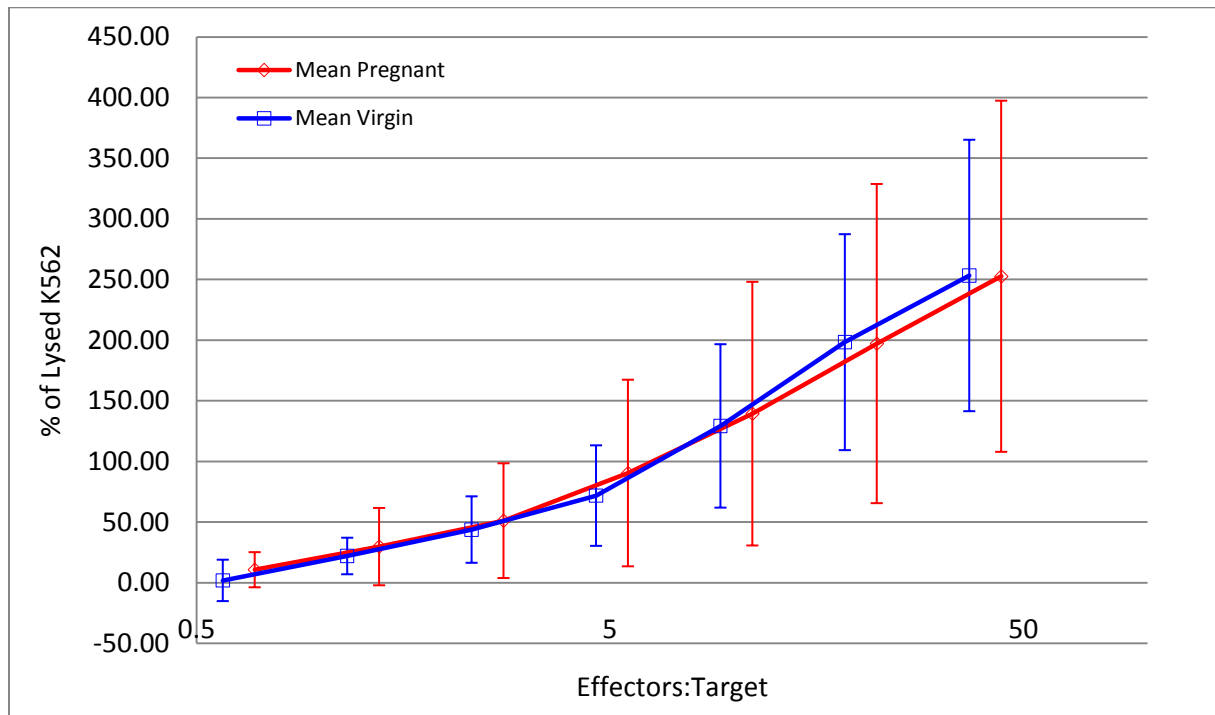


Figure 12: NK cell cytotoxicity assay. Mean of 8 32-week pregnant women blood samples compared to 8 non-pregnant blood samples.

2.2. Discussion

Even if we were not able to observe an increased number of CD33⁺ cells isolated from the PBMC layer from pregnant women, we have shown that this population is more immunosuppressive during pregnancy compared to non-pregnant state. Physiological changes related to human pregnancy may be a permissive precursor state for tumor progression, a mechanism that may be linked to reduced immunity mediated by CD33⁺ cells and by the reduced presence of NK cells. Indeed, NK cells have been shown to display reduced cytotoxicity in blood isolated from pregnant women. However, our preliminary experiments do not support the contribution of CD33⁺ cells to down-regulation of NK cell activity during pregnancy.

The main potential difficulties of this study were due to the heterogeneity of the material obtained from human origin. Although some characteristics of the blood donors were known (20-40 years old women, with no active or chronic diseases and who were 32-weeks pregnant at the time of blood sample retrieval), many aspects were not known, including the ethnic origin (for the first experiment) and the hormonal status. Furthermore, it may be more difficult to strictly identify and isolate MDSC in human compared to mice.

To explore the role of CD33⁺ cells in pregnancy in more detail and to confirm their similarity to myeloid immunosuppressive cells found in tumorigenesis, additional studies are required. Gene expression and cytokine production profile studies might help to improve the understanding of the role of these cells implicated in tumor progression and pregnancy. Although very preliminary,

our results are promising and understanding how CD33⁺ cells become more immunosuppressive during the physiological state of pregnancy and how they recover their resting stage, may lead to uncovering ways in which to reverse their activation to restore immunocompetence.

We know that human cancers are associated with restrained antitumour immunity, driven by immunosuppressive cells that participate in resistance to treatment. Thus, the CD33⁺ immunosuppressive cells may represent a therapeutic target whose function can be modulated to heighten the efficacy of conventional therapies directed against tumor cells.

CONCLUSIONS

The first aim of this work was to assess the MDSC gene expression profile to determine whether changes in MDSC features appear prior to tumour implantation and are associated with pregnancy itself. The difference between the two states (pregnancy or virginity) was reflected by sets of genes found to be differentially expressed between pregnant and virgin mouse MDSC. Pregnant mouse MDSC were associated with genes whose products contribute to ECM remodeling, tumor progression and the regulation of innate immunity. These genes may contribute to the formation of a more permissive and tumor-supportive microenvironment.

The observations were done in duplicate in two different sets of experiments and selected positively modulated genes identified during the array were validated and quantified by qRT-PCR. This work has several limitations since the number of separate experiments is too low to allow optimal quantification and statistical analysis. Furthermore, we focused our interest on overexpressed genes and we may well be missing critical genes that are marginally induced during pregnancy and/or missing genes that were repressed during pregnancy. Further work will be required to evaluate these issues but this is beyond the scope of the current master project. The latter was useful to optimize the isolation, enrichment, and RNA preparation from a heterogeneous population of cells. Large scale preparation of cells and RNAs was beyond the scope of this master project.

The second specific aim of this work was to assess MDSC activity in human pregnancy. The isolation of CD33⁺ cells from peripheral blood obtained from 16 32-week pregnant and 16 non pregnant women was successful, and these cells showed a modified function during human gestation. MDSC were more immunosuppressive in blood from pregnant women, and NK cells showed reduced numbers in blood circulation but their cytotoxic activity needs to be assessed with a new batch of samples. The changes in MDSC functions are also found in patients with cancer. Others tests will be required to evaluate and confirm this potential dysregulation of myeloid cells during human pregnancy.

Cancer-related research tries to improve the understanding of the processes implicated in tumor formation and progression with the final purpose to find effective treatments. To restore an effective immune system by targeting the MDSC population appears may be a promising approach that is worth exploring further.

Moreover, MDSCs display tumor-associated changes in their gene expression profile that may provide targets, contrary to tumor cells that are known to be associated with massive genomic alterations and drugs resistance. Thus, research efforts have to be pursued to find judicious therapeutic targets within MDSCs that can support and improve conventional therapies directed against tumor cells.

MATERIALS AND METHODS

1. In vivo experiments in mice

1.1. MDSC isolation from mice

Female C57BL/6 wild-type mice were between 8 and 10 weeks old at the beginning of experiments. Experimental procedures involving mice were approved by the Etat de Vaud, Service Vétérinaire, authorisation no. VD1477. MDSC were isolated from virgin and 16-day pregnant mice splenocytes, as well as from TBM 14-day post-subcutaneous injection of 50 000 B16-F10 syngenic melanoma cells. Briefly, spleens were extracted from euthanized mice, minced through a 100 μ m nylon mesh, washed in cold PBS and cells were resuspended in 1mL ACK (Ammonium Chloride Potassium buffer) for erythrolysis, 10 minutes at 4°C. After washing in MACS buffer (PBS-2 mM EDTA-0.5% BSA), cells were passed through a new 40 μ m nylon membrane to obtain a single-cell suspension and counted before proceeding to cell isolation according to Miltenyi MACS protocol.

1.2. Magnetic labeling

After addition to the splenocyte suspensions of FcR Blocking Reagent (blocks unwanted binding of antibodies to human Fc receptor-expressing cells, and increases the specificity of antibody labeling), the biotinylated anti-Ly-6G antibody was incubated for 10 minutes at 4°C. Cells were washed in MACS buffer and the anti-biotin-conjugated magnetic beads were added for a further 15 minutes incubation period. Samples were washed in MACS buffer, resuspended and separated on LS columns placed in the magnetic field of a MACS Separator (Miltenyi Biotec). The positive fractions (magnetically retained Ly-6G⁺ cells) were eluted, and enriched over a second LS column to increase the purity of Ly-6G⁺ cells before being washed and quantified.

1.3. RNA extraction

The RNeasy[®] Mini Kit (Qiagen, Germany) was used for total RNA extraction. RNA purification was performed according to the manufacturer's instructions: cells disruption was obtained using a lysis buffer containing β -mercaptoethanol in order to inactivate RNases. The homogenized lysate was mixed with 70% ethanol, providing optimal binding conditions, and transferred to a spin column to allow total RNA binding to the silica-based membrane of the column. Columns were washed three times, and RNA was eluted using 30 μ l RNase-free water applied directly to the spin column. Quality and quantity of RNA was assessed thanks to a NanoDrop[®] ND-1000 spectrophotometer (NanoDrop Technologies, USA).

1.4. Flow cytometry analysis of cell phenotypes

Flow cytometry (FACS for Fluorescence Activated Cell Sorting) is a method for the evaluation of cell-membrane proteins and intracellular proteins as well as peptides and DNA. Here we use it for the evaluation of cellular markers to identify the result of MDSC isolation through the magnetic separation. The principle behind FACS is an antigen-antibody reaction, with the antibodies being fluorescently labeled. The suspensions of individual cells pass a focused laser-beam. Capillary forces cause the cells to pass the flow-cell, where the labels are stimulated by the

laser light. The emitted fluorescent light from the fluorophores, which are coupled to the antibodies, and the scattered-light are detected separately.¹

The cells scatter a fraction of the light which is then detected by light detectors. The amount of light measured correlates with the size of the cells and their complexity. Granulocytes reflect more light than smooth surfaced B- or T-cells, due to their rough surface texture and a larger amount of vesicles inside the cell. A measurement for the diffraction of light in a flat angle is the forward scatter (FSC), which depends on the volume of the cell. A measurement for the diffraction of light in a right angle is the so called sideways scatter (SSC). It depends on the granularity, the size of the cells, the structure of its nucleus, and the amount of vesicles inside the cells (Figure 13).

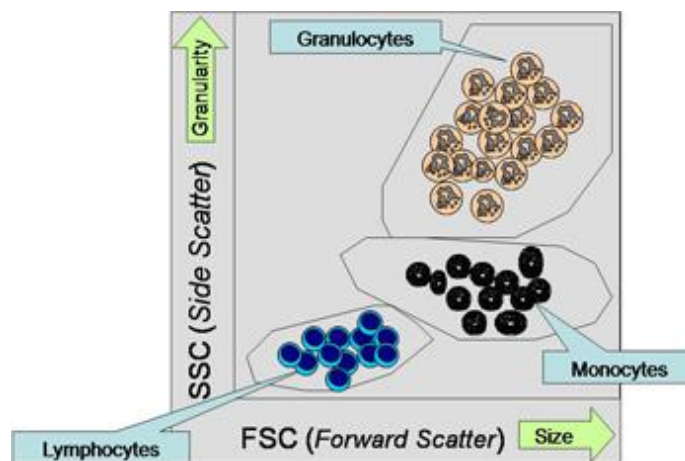


Figure 13: Characterization of unstained cells using light scatter (Dot Plot). On the right hand side: large cells. On top: granular cells. Hence, large granular cells (eg. Granulocytes) can be found on the top right, while small cells which are smooth can be found on the bottom left.

Single cell suspensions from spleen were prepared as described above and erythrolysed. Cells were counted, 10^6 cells aliquoted into FACS tubes and washed once with FACS buffer (PBS-3%FBS-0.9%NaN₃). Staining was done in a final volume of 50 μ l for 45 minutes at 4°C. Antibody dilutions were as follows: CD45-FITC (1:200), CD11b-PE (1:300), Gr1-APC (1:200) (all from BioLegend). After washing, cells were fixed in 1% paraformaldehyde in PBS for 10 minutes at 4°C, washed in FACS buffer and resuspended in the same buffer for an immediate acquisition in a FACS Gallios (Beckman Coulter). Kaluza software was used for image analysis and quantification of cell populations.

1.5. Microarray study

The quality of the total RNA is essential for the overall success of the analysis. The steps of fragmentation, hybridization, washing and scanning were performed at the DNA Array Facility of Lausanne (DAFL, <http://www.unil.ch/dafl>). In the present study, the GeneChip Mouse Gene 1.0ST arrays (Affymetrix, UK) were used, representing approximately 29,000 different RNAs,

¹<http://www.antibodies-online.com/resources/17/607/Flow+cytometry+FACS+Principle+and+experimental+setup/>

which cover almost all genome (Figure 14). Gene expression data normalization and subsequent analysis of differentially expressed genes were done by Professor Paolo Provero at the University of Turin, using Bioconductor.

The obtained lists were sorted out according to the estimate of the log₂-fold-change of expression (log₂FC). This value allowed the selection of the most biologically relevant probe sets. Genes of interest were selected among the probe sets with log₂FC ≥ 1.41, what insured to have more than 2-fold-change of expression (log₂FC = log₂ (Pregnant) - log₂ (Virgin)).

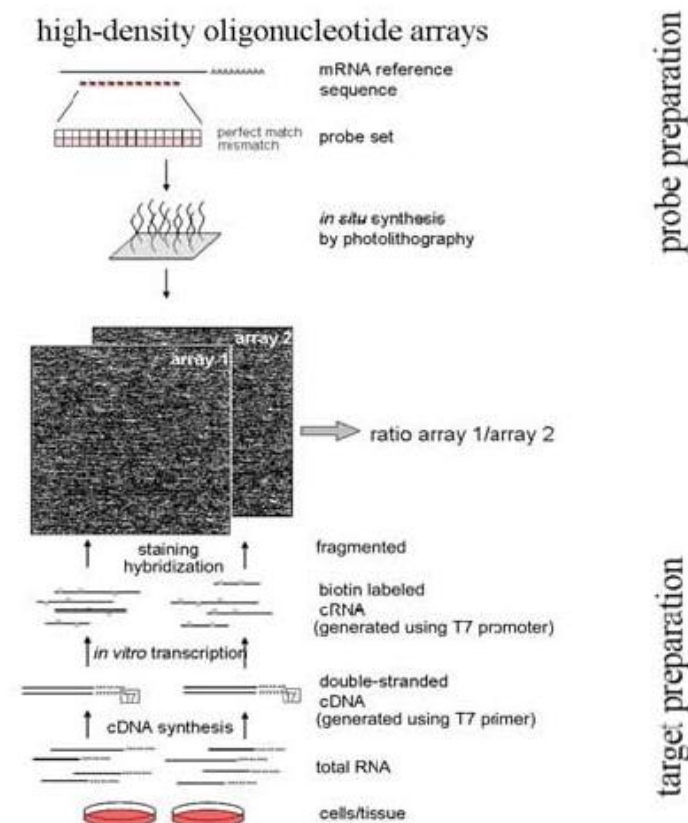


Figure 14: Overview of steps needed for Affymetrix microarray hybridization².

1.6. Quantitative real-time RT-PCR validation of microarray results

5 up-regulated genes identified in the microarray study were chosen in order to validate the experiment using quantitative real-time reverse transcription-polymerase chain reaction (qReal-time RT-PCR), that allowed quantitative assessment of gene expression levels. The same quantity (500 ng) of total RNA molecules was first transcribed to cDNA in a reverse transcription (RT) step.

²<http://compbio.pbworks.com/w/page/16252906/Microarray%20Normalization%20and%20Expression%20Index#Microarrayrecap>

The cDNA served as template for the PCR step during which specific region of a gene of interest (Table 2) is amplified using gene-specific primers and probes labeled with a fluorescent dye. The qReal-time RT-PCR allows the monitoring of the amount of a product by detecting the increase of fluorescence intensity released during the PCR reaction. Knowing the amplification efficiency of the PCR, which typically is close to a doubling of the number of molecules per amplification cycle, it is then possible to evaluate the initial amount of RNA molecules, i.e. the level of expression of a particular gene present in a sample. The fluorescence intensity is produced by Syber green dye. Syber green intercalates only in the double-stranded DNA (such as PCR products obtained during the amplification step) and thus it increases along with the increase of the amount of the PCR product. Gene expression was normalized to a housekeeping gene (GAPDH).

Table 2: Primers used for the qRT-PCR	
Fibronectin-1 Amplicon Size = 87 Forward Reverse	AGCCCGGATGTTAGAAGCTA GGCATTGTCGTTTCAGAGTGT
Ficolin B Amplicon Size = 83 Forward Reverse	TGTGACATGGACACAGATGG GTCCAGTCCCAGAAAGAAGTC
Inhibin Beta-A Amplicon Size = 84 Forward Reverse	CTCGGAGATCATCACCTTTG GACAGGTCACCTGCCTTCCTT
Chitinase 3-like 3 (Chi3l3) Amplicon Size = 95 Forward Reverse	TGGTGAAGGAAATGCGTAAA GTCAATGATTCTGCTCCTG
Olfactomedin 4 Amplicon Size = 129 Forward Reverse	TGCTCGAAGTGGAGATAAGG AGGCCTCACATTCTTTCAGC
GAPDH Amplicon Size = 174 Forward Reverse	ACCACCAACTGCTTAGCCCCCTGGCCAA GGATGCAGGGATGATGTTCTGGGCAGCCCCAC

2. In vitro experiments with human cells

We required some preliminary experiments and we used buffy coats in order to develop protocols and isolate NK and T cells for the following studies. CD56 MicroBeads were used for the positive selection of Natural Killer cells from human PBMC and CD3 MicroBeads for T cell isolation (Miltenyi Biotec).

2.1. Samples

Whole blood samples were collected from sixteen 32-weeks pregnant and sixteen non-pregnant women. Informed consent was obtained and ethical approval for the study was granted by the Institutional Ethics Committee. The blood was collected into tubes containing EDTA anticoagulant.

2.2. Isolation of mononuclear cells and granulocytes from human peripheral blood by density gradient centrifugation

Human PBMC and granulocytes were separated from peripheral blood of donors by gradient centrifugation on Ficoll-Hypaque (Lymphoprep) at room temperature, according to Miltenyi MACS protocol. The blood samples were diluted with 2-4X the volume of buffer (PBS-2 mM EDTA) then 20 mL of diluted cell suspension were carefully layered over 6 mL of Ficoll-Paque in a 50 mL conical tube. Differential migration during centrifugation (30 minutes, 2000 rpm, 20°C, without brake) results in the separation of cell types into different layers (Figure 15). The bottom layer contains Ficoll-aggregated red blood cells. Immediately above this is a diffuse layer containing mostly granulocytes and unbound Ficoll®. Due to a slightly lower density, the lymphocytes (including the monocytic PBMC fraction) sediment at the interface between the Ficoll® and uppermost plasma/platelet layer.

PBMC were removed from the interface and subjected to multiple washes in buffer to remove any residual Ficoll. The granulocyte layer was therefore collected and resuspended in 8 mL ACK for erythrolysis, 10 minutes at 4°C before washing. The cell suspensions were resuspended and counted before proceeding to cell isolation according to Miltenyi MACS protocol.

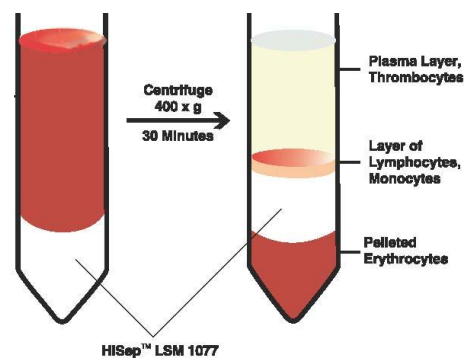


Figure 15: Differential migration following centrifugation results in the formation of several cell layers⁷.

2.3. Magnetic labeling

The magnetic labeling was performed on the PBMC and granulocyte layers. The method used was the same as described above for the mouse side of experiments, but with CD33 or CD11b Microbeads, in order to isolate a myeloid cell population. The cell pellets were resuspended in 70 μ L of buffer per 10^7 total cells and 10 μ L of Human FcR Blocking. Then we added 20 μ L of CD33/CD11b MicroBeads per 10^7 total cells for 15 minutes incubation at 4°C. Samples were washed in MACS buffer, resuspended and separated on LS columns placed in the magnetic field of a MACS Separator (Miltenyi Biotec).

2.4. FACS analysis

Single cell suspensions were prepared as described above (mice experiments). Antibodies used were as follows (1:20 dilution): CD3-PC7, CD11b-APCeF1780, HLA-DR-APC, CD33-eFluor450 and CD56-ECD.

2.5. CD33⁺ cells and T cells co-culture: ³H-Thymidine T cells proliferation assay

To follow the immunosuppressive function of the CD33⁺ cells, they were co-cultured with T cells and plated in a 96-well plate before to add ³H-Thymidine. During each cell division the cell will incorporate ³H-Thymidine into its DNA. The more cell divisions (or the higher the proliferation rate) the more radioactivity will be incorporated into DNA.

T cells from one unique buffy coat and CD33⁺ cells from non-pregnant and pregnant woman bloods were mixed in a ratio of 1:1, 1:0.5, 1:2 and 1:4, everything in triplicate, and co-cultured in RPMI-1640 plus 10% FBS in a 96-well plate in the presence of anti-CD3/anti-CD28-coated beads (Dynal Biotech) at a 1:1 ratio for T cell stimulation. For ³H-thymidine proliferation assays, ³H-T was added (1 curie/well) after 4 days of co-culture. 18 hours later, the co-culture was stopped by frizzing the plate until harvesting cells were washed out of the 96-well plates with water and collected on a filter membrane. After drying, the amount of radioactivity was counted in a scintillation counter, after adding 25 μ L of MicroScint per well. Comparison of the counted radioactivity (counts per minute = cpm) of cells allows to evaluate the T cell proliferation in presence of different concentrations of immunosuppressive cells.

2.6. NK-CD33⁺ co-culture

MACS-isolated NK cells from one unique human buffy coat and CD33⁺ extracted from eight non-pregnant and eight pregnant women blood samples (one half of the total samples) were mixed at 1:1, 1:2, 1:4 ratios (typically 1×10^4 cells each) and co-cultured for 18h in RPMI-1640-10%FBS-1%P/S and 500 ng/ml recombinant human IL-2 (in order to activate NK cells) in a 24-well plate. Cells were collected, counted and aliquoted in triplicates into 96-well plates for ⁵¹Cr-release cytotoxicity assay on the tumor cell line K562 (NK-sensitive human erythromyelocytic leukemia cell line), as described in the following point.

2.7. ⁵¹chromium-release cytotoxicity assay

We used the negative fraction post-CD33 sorting which should be enriched in NK cells, as effectors, maintained overnight at 37°C in complete RPMI + hIL-2 before plating.

Single cell suspensions were counted in leukoplate and aliquoted into triplicate wells of conical-bottom 96-well plates. Starting dilutions were 200:1 and titration was done through serial dilution (3:1) into the next 5 wells. As target cells, we used the cell line K562. They were maintained in RPMI and split to $3 \cdot 10^5$ cells/ml 2 days before to testing to ensure that cells are in log phase. On the day of testing, cells were washed in PBS and 10^6 cells were labelled with $^{51}\text{CrO}_4$ for 1h at 37°C . Labelled cells were washed extensively in complete medium, resuspended in complete RPMI, counted and plated with several ratios Effector:Target. After 4h of incubation at 37°C , ^{51}Cr release into the media was quantified using LumaPlate-96 plates (PerkinElmer) and a Top-Count- γ -counter (PerkinElmer). 0% lysis was determined with complete medium only, 100% lysis by adding 100 μl of 1 M HCl to the target cells. Percentage of lysis was determined by the following equation: $100 \times (\text{sample lysis} - 0\% \text{ lysis}) / (100\% \text{ lysis} - 0\% \text{ lysis})$. NK cell proportions of each sample were determined by flow cytometry and Effector:Target ratios adjusted accordingly.

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